MOLECULAR MARKER ASSOCIATED WITH CMV RESISTANCE AND USE THEREOF

TECHNICAL FIELD

The present invention relates to a cucumber mosaic virus (CMV) resistance-associated molecular marker and the use thereof, and more particularly to a nucleic acid consisting of nucleotide sequences having a very high association with the CMV-resistant character of plants, a primer comprising a portion of the nucleotide sequence of the nucleic acid, and a method for detecting CMV-resistant plants using the nucleic acid or the primer.

BACKGROUND ART

Cucumber Mosaic Virus (hereinafter, referred to as "CMV") is a plant-pathogenic virus having the widest host range of plant viruses, and causes great economic damage to about 900 kinds of dicotyledonous and monocotyledonous plants in the whole world. Plants infected with CMV show mosaic symptoms on leaves, stems and fruits. Particularly, the affected leaves become smaller in size and crumpled, and show lesions along leaf veins. Also, the affected fruits show dark-green mosaic patterns and become uneven, thus reducing product quality.

Meanwhile, in order to breed a disease-resistant variety, a disease-resistant factor should be introduced by successive backcrossing from other varieties having the factor. In each of the introduction steps, the disease-resistant factor should be selected through a resistance test, and in this selection step, the use of a molecular marker having a close association with the disease-resistant factor will make the selection very convenient. Thus, methods for the diagnosis of CMV using the

molecular marker and various technologies for the development of CMV-resistant varieties by transformation have been developed. For example, Korean Patent Application No. 2000-0025699 discloses the nucleotide sequence of a set of gene amplification primers for the diagnosis of cucumovirus, in which the primer set allows the diagnosis of CMV, peanut stunt virus and tomato aspermy virus, which belong to the cucumovirus group, as well as a method for the diagnosis and identification of genes using the primers. Also, Korean Patent Registration No. 0293567 discloses a method for developing CMV-resistant tomato lines, which comprises transforming tomatoes with a CMV coat protein gene isolated in Korea. Furthermore, Korean Patent Application No. 1993-0029605 discloses a hammerhead-type ribozyme which attacks the RNA of a CMV coat protein gene, in order to produce transgenic crops having resistance to CMV causing diseases in crops.

As described above, the prior art on the diagnosis of CMV resistance and on the development of CMV-resistant plants targets the CMV coat protein gene, and there is no report of the development of technology concerning a CMV-resistant factor which is inherent in plants.

Accordingly, there has been an urgent need for the development of a CMV resistance-associated molecular marker from plant lines having the CMV-resistant factor and for the development of a method for diagnosing CMV infection in plants using the developed molecular marker.

Disclosure of the Invention

Therefore, the present invention has been made to satisfy the above-mentioned need, and it is an object of the present invention to provide a molecular maker consisting of nucleotide sequences having a very high association with a CMV-

resistant character, and the use thereof.

To achieve the above object, in one aspect, the present invention provides an isolated nucleic acid consisting of a nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22.

In another aspect, the present invention provides a primer for the detection of CMV-resistant plants, which comprises consecutive nucleotides selected from a nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22.

In still another aspect, the present invention provides a kit for detection of CMV-resistant plant, which comprises the nucleic acid or the primer.

In yet another aspect, the present invention provides a method for detecting a CMV-resistant plant and a method for determining the genotype of the plant, the methods comprising analyzing the genomic DNA of a plant in the presence of the nucleic acid or the primer.

The further another aspect, the present invention provides a CMV-resistant plant which reproduce asexually by tissue culture and comprises a nucleic acid consisting of a nucleotide sequence shown SEQ ID NO: 2 or SEQ ID NO: 22, as well as a seed obtained therefrom.

Hereinafter, the present invention will be described in detail.

The present invention provides a molecular marker having a high association with the CMV-resistant character of plants (hereinafter, referred to as "a CMV resistance-associated molecular marker"). The CMV resistance-associated molecular marker provided in the present invention comprises a nucleic acid consisting of a nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22. The nucleic acid

includes RNA, DNA and cDNA, and preferably means DNA. The inventive molecular marker consists of nucleotide sequences having a close association with a CMV-resistant character, and is placed significantly close to the CMV-resistant gene. For this reason, the use of any polymorphism shown by the nucleotide sequence allows the presence or absence of the CMV resistance in plants to be determined.

Also, the inventive molecular marker comprises a primer for the detection of CMV-resistant plants, which comprises a series of nucleotides selected from the nucleotide sequence of the nucleic acid. The primer may preferably have at least 10 consecutive nucleotides, and more preferably at least 12 consecutive nucleotides, which are selected from the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22. The inventive primer can be so designed that it is suitable for various DNA polymorphism analyses known in the art, such as RFLP (restriction fragment length polymorphism), RAPD (randomly amplified polymorphic DNA), DAF (DNA amplification fingerprinting), AP-PCR (arbitrarily primed PCR), STS (sequence tagged site), EST (expressed sequence tag), SCAR (sequence characterized amplified regions), ISSR (inter-simple sequence repeat amplification), AFLP (amplified fragment length polymorphism), CAPS (cleaved amplified polymorphic sequence), PCR-SSCP (single-strand conformation polymorphism) and the like (Jordan *et al., Theor. Appl. Genet.*, 106:559-567, 2003; Martins M., R. *et al., Plant Cell Reports*, 22:71-78, 2003; Williams, J. G. K. *et al., Nucl. Acids Res.*, 18:6531-6535, 1990; Michelmore, R. W., *et al., Proc. Natl. Acad. Sci.* USA. 88:9828-9832, 1991; Martins *et al., Plant Cell Rep.*, 22:71-79, 2003; Orita *et al., Proc. Natl. Acad. Sci.* USA 86:2766-2770, 1989). For the DAF analysis, a primer consisting of 5-8 consecutive nucleotides can be designed and used. For example, a primer for the STS analysis can be so designed that it coincides with the terminal nucleotide sequence of an RFLP

marker, and a primer for the SCAR analysis can be designed based on the terminal nucleotide sequence of an RAPD marker. Also, a primer for the CAPS analysis can be so designed that it contains a suitable restriction site. Preferably, the primer provided in the present invention may have a nucleotide sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 23 to SEQ ID NO: 26. Furthermore, the inventive primer may have modifications (e.g., additions, deletions and/or substitutions) if the modifications have no effect on the detection of polymorphism associated with a CMV-resistant character. Preferably, the inventive primer can be modified by making an addition and/or substitution of any base in at least 12 consecutive nucleotides selected from the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22. For example, the inventive primer may have a nucleotide sequence of SEQ ID NO: 27 or SEQ ID NO: 28.

The inventive molecular marker can be very useful for the detection of CMV-resistant plants. Thus, the present invention provides a method for the detection of a CMV-resistant plant, which comprises analyzing the genomic DNA of a plant in the presence of the nucleic acid or the primer. This method may be performed using various DNA polymorphism analyses known in the art. Example of the DNA polymorphism analyses which can be used in the present invention include, but are not limited to, RFLP (restriction fragment length polymorphism), RAPD (randomly amplified polymorphic DNA), DAF (DNA amplification fingerprinting), AP-PCR (arbitrarily primed PCR), STS (sequence tagged site), EST (expressed sequence tag), SCAR (sequence characterized amplified regions), ISSR (inter-simple sequence repeat amplification), AFLP (amplified fragment length polymorphism), CAPS (cleaved amplified polymorphic sequence), PCR-SSCP (PCR- single strand conformation polymorphism) and the like (Jordan *et al., Theor. Appl. Genet.*, 106:559-567, 2003;

Martins M., R. *et al.*, *Plant Cell Reports*, 22:71-78, 2003; Williams, J. G. K. *et al.*, *Nucl. Acids Res.* 18:6531-6535, 1990; Michelmore, R. W., *et al.*, *Proc. Natl. Acad. Sci.* USA. 88:9828-9832, 1991; Martins *et al.*, *Plant Cell Rep.*, 22:71-79, 2003; Orita *et al.*, *Proc. Natl. Acad. Sci.* USA 86:2766-2770, 1989). Preferably, the detection method may be performed by the RFLP, RAPD or CAPS analysis.

Concretely, the detection method may be performed by the CAPS analysis comprising the steps of:

(a) performing PCR on each of genomic DNA templates obtained from CMV-resistant plants and CMV-susceptible plants, using a primer comprising a series of nucleotides selected from the nucleotide sequence set forth in SEQ ID NO. 2 or SEQ ID NO. 22;

(b) digesting the PCR product with a restriction enzyme;

(c) electrophoresing the digested DNA fragments on agarose gel; and

(d) performing the comparison between the DNA band patterns of the electrophorsed gel.

The primer in the step (a) may preferably be selected from the group consisting of SEQ ID NO. 23 to SEQ ID NO: 28. Preferably, a primer combination selected from the group consisting of a combination of SEQ ID NO: 23 and SEQ ID NO: 24, a combination of SEQ ID NO: 23 and SEQ ID NO: 25, a combination of SEQ ID NO: 23 and SEQ ID NO: 26, and a combination of SEQ ID NO: 27 and SEQ ID NO: 28, may be used. Furthermore, as the restriction enzyme in the step (b), any restriction enzyme present in the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22 may be used without limitations, and preferably, *Xba*I or *Eco*RI may be used.

Moreover, the detection method may also be performed by the RAPD

analysis comprising the steps of:

(a) performing PCR on each of genomic DNA templates obtained from CMV-resistant plants and CMV-susceptible plants, using a primer capable of amplifying the nucleotide sequence set forth in SEQ ID NO: 2;

(b) electrophoresing the PCR products on agarose gel; and

(c) performing the comparison between the DNA band patterns of the electrophoresed gel.

The primer in the step (a) may have all nucleotide sequences which can be designed by a person skilled in the art so as to amplify the nucleic acid set forth in SEQ ID NO: 2. Preferably, the primer may have a nucleotide sequence set forth in SEQ ID NO: 1.

In addition, the detection method may also be performed by the RFLP analysis comprising the steps of:

(a) digesting each of genomic DNA templates obtained from CMV-resistant plants and CMV-susceptible plants with a suitable restriction enzyme;

(b) electrophoresing the digested DNA fragments on agarose gel;

(c) transferring the DNA-containing gel to a nylon membrane;

(d) performing Southern blot analysis for the gel using the nucleic acid having the nucleotide sequence set forth in SEQ ID NO: 2 as a probe; and

(e) exposing the gel onto an X-ray film to perform the comparison between the DNA band patterns of the gel.

As the restriction enzyme in this method, any restriction enzyme known in the art may be used without limitations, and preferably a restriction enzyme causing DNA polymorphism may be used. More preferably, an enzyme selected from the group consisting of *Eco*RI, *Eco*RV and *Xba*I may be used.

Also, the inventive molecular marker may be used to determine the genotype of the CMV-resistant plant. Thus, the present invention provides a method for determining the genotype of a CMV-resistant plant, which comprises analyzing the genomic DNA of a plant in the presence of the inventive nucleic acid or primer. This method may be performed by various DNA polymorphism analyses known in the art. Concretely, this method is performed as described for the method for detecting the CMV-resistant plants.

The plant to which the inventive methods may be applied includes, but is not limited to, cucumber, watermelon, red pepper, melon, Chinese cabbage, tobacco, Petunia, cotton, and rose.

Moreover, the present invention provides a kit for the detection of a CMV-resistant plant, which comprises either a nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22, or a series of nucleotides selected from the nucleotide sequence of the nucleic acid. The inventive kit may additionally comprise various reagents required not only in test procedures (PCR, Southern blot analysis, etc) to detect CMV-resistant plants using the above nucleic acid or primer but also in a procedure to examine the test results. For example, the inventive kit may additionally comprise PCR reaction mixture, restriction enzyme, agarose, buffer required for hybridization and/or electrophoresis, etc.

Furthermore, the present invention provides a CMV-resistant plant which comprises a nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22, the nucleic acid being a molecular marker having a close

association with the CMV resistant character of plants. As used herein, the term "a CMV-resistant plant" refers to the plant showing a character of resistance to CMV. The plant includes, but is not limited to, cucumber, watermelon, red pepper, melon, Chinese cabbage, tobacco, Petunia, cotton and rose. The red pepper is preferable. Also, the CMV-resistant plant provided in the present invention includes the organ, tissue, cell, seed and callus of plants. Tests on whether the CMV resistance of the inventive CMV-resistant plants is hereditary were performed, and the test results proved that the CMV resistance is determined by a dominant single gene. The inventive CMV-resistant plant can reproduce asexually by a general tissue culture method known in the art. For example, the inventive plant can reproduce asexually by fine reproduction by organ generation (e.g., a method of culturing tissue, such as leaves having no organ formed therein, leaves, petioles, stem nodes, cotyledons, cotyledon axes or the like, to induce fresh shoots on the surface of the tissue) or regeneration by callus induction, and the like. In addition, the present invention provides a seed which is obtained from the CMV-resistant plant and comprises the nucleic acid consisting of the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 22.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a photograph showing the results of RAPD analysis performed using operon primers (OPC-04 to OPC-08 and OPC-10) and using CMV-resistant DNA pool (R) or CMV-susceptible DNA pool (S) as templates.

FIGS. 2a and 2b are photographs showing the results of RAPD analysis performed for resistant plants (R_0) and their F1 (R_1), susceptible plants (S_0) and their F2 (S_1), resistant plants F2 and susceptible plants F2, using an OPC-07 primer (SEQ

ID NO: 1).

FIG. 3 shows the results of RFLP analysis performed using a DNA fragment (SEQ ID NO: 2) amplified by an OPC-07 primer as a probe.

R: resistant plants

S: susceptible plants

FIG. 4 is a photograph showing the results of CAPS analysis performed using primers of SEQ ID NO: 23 and SEQ ID NO: 24.

FIG. 5 is a photograph showing the results of CAPS analysis performed using a primer combination of SEQ ID NO: 23 and SEQ ID NO: 25.

A: digested with *Eco*RI

B: digested with *Xba*I

FIG. 6 is a photograph showing the results of CAPS analysis performed using a primer combination of SEQ ID NO: 23 and SEQ ID NO: 26.

FIG. 7 is a photograph showing the results of CAPS analysis performed using a primer combination of SEQ ID NO: 27 and SEQ ID NO: 28.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be described in further detail by the following examples. It is to be understood, however, that these examples are given for illustrative purpose only and are not intended to limit the scope of the present invention.

Example 1: Construction of crossed population for molecular marker development of CMV-resistant red pepper plants and examination of hereditary pattern of CMV resistance

CMV was inoculated into a variety of red pepper plants so as to screen CMV-resistant plants. The results showed that one plant of the elgiencho lines was CMV-resistant. The screened plant was named "FP11". In order to test whether the CMV resistance of the screened FP11 is hereditary or not, a self-pollinated population and a crossed population were constructed. The crossed population was constructed by crossing the plant FP11 with FP14, a susceptible line. The crossed F1 seeds were sowed and self-pollinated to produce the F2 population, and the F2 plants were self-pollinated to produce F3 plants. The F2 and F3 populations were tested for CMV resistance, and the results showed that the CMV resistance was hereditary at a ratio of 3:1 in the F2 population, and the ratio of homo to hetero in the F3 generation was 1:2. This suggests that the CMV resistance is determined by a dominant single gene.

Example 2: DNA isolation from plants

DNA extraction was performed by a modification to the method of Prince, J. P., *et al.*(Prince, J. P., *et al.*, *HortScience* 32:937-939, 1997). Red pepper leaves stored at –80 °C were crushed with liquid nitrogen, and then well mixed 25 ml of DNA extraction buffer (7M urea, 0.35M sodium chloride, 0.05M Tris-HCl pH 8.0, 0.02M EDTA, 0.25% sarkosyl, 5% phenol, 0.2% sodium bisulfate). Thereafter, the mixture was introduced with 0.75 ml of 20% SDS, and incubated at 65 °C for 30 minutes while shaking at intervals of 10 minutes.

A solution of chloroform and isoamyl alcohol (24:1) was filled to the end of a 50-ml tube and well mixed for 15 minutes. The mixture solution was centrifuged at 5,000 rpm for 15 minutes, and the obtained supernatant was transferred into a fresh 50-ml tube using cheesecloth. Next, the same volume of isopropanol was added to the tube and mixed, followed by the precipitation of DNA for 1 hour. The

precipitated DNA was collected using an U-shaped Pasteur pipette and placed in a 1.5-ml micro-tube to which 70% ethanol was then added to reprecipitate the DNA. The resulting DNA was dried at room temperature for 30-40 minutes. To the dried DNA pellet, 600-700 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA) was added, and the DNA solution was incubated at 65 °C for 1 hour. The culture solution was centrifuged three times. Then, the resulting DNA was transferred into a 1.5-ml tube and added with RNase (100 μg/ml) so as to remove RNA. The final concentration of the purified DNA was measured with a fluorometer.

Example 3: Selection of CMV resistance-associated RAPD primer

In order to select a DNA molecular marker associated with a CMV-resistant character, RAPD (Williams, J. G. K. *et al., Nucl. Acids Res.* 18, 6531-6535, 1990) and BSA (Bulked Segregant Analysis) (Michelmore, R. W. *et al., Proc. Nat. Acad. Sci.*, 88:9828-9832, 1991) were used. Based on the CMV resistance assay results from the F2 population of Example 1, a DNA pool of 10 resistant F2 plants and a DNA pool of 10 susceptible F2 plants were prepared, respectively. The each DNA pool was controlled to a DNA concentration of 20 ng/μl. Then, the PCR was performed using the DNA as a template. In this case, for RAPD primers, about 147 primers causing a good PCR reaction were selected from about 400 primers of Operon RAPD 10-mer kits series A to series T (Operon, Alameda, CA, USA), and the selected primers were used to probe bands showing a specific difference in amplification between the resistant DNA pool and the susceptible DNA pool.

The PCR reaction mixture consisted of a 25-μl total volume of 1 X PCR buffer, 60 ng of the template DNA, 0.3 mM dNTP, 0.6 pM primer, 3.5 mM $MgCl_2$, and 0.6 Unit Taq DNA polymerase (Takara, Japan). The PCR amplification

consisted of the following: denaturation of template DNA of 4 minutes at 94 °C; 45 cycles each consisting of 1 minute at 94 °C, 1 minute and 30 seconds at 36 °C, and 1 minute and 50 seconds at 72 °C; and final extension of 5 minutes at 72 °C. As a result, as shown in FIG. 1, a molecular marker which had show specificity only to the resistant pool in the PCR reaction with an OPC-07 primer (SEQ ID NO: 1) was selected.

In order to examine whether the molecular marker is linkaged to a CMV-resistant character, RAPD analysis was further performed on a resistant plant and a susceptible plant and their F1 generation, and 183 resistant F2 plants and 64 susceptible F2 plants. As shown in FIGS. 2a and 2b, the analysis results showed that the molecular marker was 100% co-segregated.

Example 4: Cloning and nucleotide sequence determination of RAPD molecular marker

The DNA amplified with the OPC-07 primer (10 mer) having a nucleotide sequence set forth in SEQ ID NO: 1 was isolated and purified from gel, and cloned into a pGEM-T vector (Promega). The cloned plasmid was introduced into *E. coli* DH 10B by electroporation, and transformants were screened in an antibiotic-containing medium. Recombinant plasmids were isolated from the screened transformants, and the nucleotide sequences of DNAs contained in the plasmids were analyzed. The determined nucleotide sequences are set forth in SEQ ID NO: 2.

Example 5: Southern blot analysis for inverse PCR and conversion into CAPS molecular marker

The genomic DNA of each of resistant plants and susceptible plants was

prepared, and digested with DNA restriction enzymes, *Eco*RV, *Hin*dIII, *Xba*I and *Eco*RI, respectively. The digested DNA was electrophoresed on agarose gel. The DNA was transferred to a nylon membrane and then subjected to Southern blot analysis using a CMV-resistant specific DNA probe (SEQ ID NO: 2) amplified in Example 4. The analysis results are shown in FIG. 3. As shown in FIG. 3, DNA polymorphism was shown for *Eco*RI, *Xba*I, *Eco*RV but was not shown for *Hin*dIII. Based on the RFLP analysis results, *Eco*RI, *Xba*I and *Eco*RV restriction enzymes were used for inverse PCR and conversion into a CAPS molecular marker.

Example 6: Determination of nucleotide sequence of DNA close to CMV-resistant gene

Based on the results of Example 5 shown in FIG. 3, the parts of the nucleotide sequence of the RAPD molecular marker analyzed in Example 4 were used to construct primers. The nucleotide sequence of each of the constructed primers is given in Table 1 below.

Table 1: Primers used in inverse PCR

Primer name	Nucleotide sequence (5'→ 3')	SEQ ID NO
CRSCC07a	GTC CCG ACG ATA GCC CAA AAG	3
CRINVR65	TTG GCC CTA TGA GTC CGT AC	4
CRINVR125	ACT GAC TAC GAG TTG TCA CC	5
CRINVF629	TAG GGG TTC AAG GAT CAC CC	6
CRINVR796	TAT CCT CTT ATG CAA TGC GC	7
CRINVR840	AAT CCT TGT ACC TCA CAA CG	8

CRINVF975	CGA TGC CAC TTC ATA ATG CC	9
Inv l030514 R	GAC TTG GGC ACT ACA CTG GA	10
Inv l030514 F	ACA TAG GCG TGT GCT CTG GA	11
CR 1541-3	GGA GTT TCA TCA TAT GAA GCC	12
InvXbTopF1010	GGT TCA AGG ATC ACC CAA ATA A	13
InvXbTopR107	TTC ACC TTA GTC CCC AAA CCT A	14
EV Inver F2	AAC CCA AGC CTA TTT TAG CC	15
EV-INV-XbaI	GGT AAT AGG GTT CAC CTT AGT C	16
CRINVF5095	CTT TGA GCC AAA GAA TGG AA	17
CRINVR4776	TTT GGT AAT GAC CGG AGA CC	18
INVER0827R	ATA GCA GAG GAG CAC CCT AC	19
INVER0827F1	GGT ACA AGG ATT CCC CAA AGT G	20
INVER0827F2	GAT TTA GTC AGT ATG ACG ATG CCA C	21

The primers set forth in Table 1 above were used to perform inverse PCR on resistant plants according to a method known in the art (Sambrook J. and D. W. Russel. 2001. Molecular Cloning. 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

First, the genomic DNA digested with *Hin*dIII was circularly ligated with T7 DNA ligase. The ligated DNA template was then amplified by PCR with a primer combination Of SEQ ID NO: 5 and SEQ ID NO: 6. The PCR product was reamplified by nested PCR using a primer combination of SEQ ID NO: 4 and SEQ ID NO: 9, thus obtaining a PCR product with about 900-bp size. The nucleotide sequence of the PCR product was analyzed by a primer walking method. The nucleotide sequence was linked to a nucleotide sequence of SEQ ID NO: 2, thus

obtaining a 1.8-kb nucleotide sequence having *Hin*dIII sites at both ends. Thereafter, based on the 1.8-kb nucleotide sequence, primers of SEQ ID NO: 10 and SEQ ID NO: 11 were constructed. The primer combination of SEQ ID NO: 10 and SEQ ID NO: 11 was used to perform inverse PCR using a genomic DNA template which had been digested with *Eco*RV and circularly ligated. The nucleotide sequence of the PCR product was linked to the 1.8-bp nucleotide sequence, thus obtaining a 3.4-kb nucleotide sequence having *Eco*RV sites at both ends. Similarly, a genomic DNA template which had been digested with *Xba*I and circularly ligated was subjected to inverse PCR with primers of SEQ ID NO: 13 and SEQ ID NO: 14. The nucleotide sequence of the PCR product was analyzed and linked to the 3.4-kb nucleotide sequence. As a result, a nucleotide sequence with about 5.6-kb length containing a nucleotide sequence of SEQ ID NO: 2 was determined. This nucleotide sequence is set forth in SEQ ID NO: 22.

Example 7: Conversion into CAPS molecular marker

After finding out the DNA restriction enzyme site of DNA nucleotide sequences known in the nucleotide sequences of SEQ ID NO: 22, the restriction enzyme site of genomic DNA of resistant plants was compared with that of susceptible plants. Then, *Xba*I (T'CTAG_A), *Eco*RI (G'AATT_C) and *Eco*RV (GAT'ATC), which are restriction enzymes showing the polymorphism between the two genomic DNAs, were selected as enzymes to be used in CAPS. Then, primers capable of amplifying DNA fragments showing a restriction pattern with suitable length. Namely, 4 primers each consisting of a series of nucleotide sequences selected from the nucleotide sequences set forth in SEQ ID NO: 22 were constructed (see Table 2 below).

Table 2: Primers for development of CAPS molecular marker for examination of CMV resistance

No	Nucleotide sequence (5'→ 3')	Primer name	Direction	SEQ ID NO
1	GTAGTAGGGTACGGACTCATA	SCC07S3	Forward	23
2	GTCCCGACGATAGCCCAAAAG	SCC07a	Reverse	24
3	GGAGTTTCATCATATGAAGCC	CR1541-3	Reverse	25
4	AGTGGAGCTTGGGGTAGTCC	FP5416R	Reverse	26

The genomic DNA was extracted from a resistant plant, a susceptible plant and their F2 population, respectively, and used as a template for PCR. The PCR was performed with the DNA template and a primer combination of SEQ ID NO: 23 and SEQ ID NO: 24, a primer combination of SEQ ID NO: 23 and 25, and a combination of SEQ ID NO: 23 and 26, respectively. The PCR amplification consisted of the following: initial denaturation of template DNA of 1 minute at 94 °C; 35 cycles each consisting of 1 minute at 94 °C, 1 minute at 58 °C, and 2 minutes at 72 °C; and final extension of 10 minute at 72 °C. Thereafter, each of the amplified DNAs was digested with restriction enzymes (*Eco*RI, *Xba*I, *Eco*RV) selected in Example 6, and electrophoresed on agarose gel so as to examine if the DNA was divided into genotypes, i.e., homo (RR) and hetero (Rr).

The results of the PCR amplification performed with the primer combination of SEQ ID NO: 23 and SEQ ID NO: 24 are shown in FIG. 4. The PCR-amplified DNA fragment was 1.0 kb in size. The PCR-amplified DNA fragment was digested with *Xba*I, and the result showed that the band patterns of the susceptible and resistant

plants were different from each other. Also, it could be fount that these band patterns were co-segregated with the genotype of the F2 plants, which had been expected by testing the disease resistance of F3 in Example 1.

The results of the PCR amplification performed with the primer combination of SEQ ID NO: 23 and SEQ ID NO: 25 are shown in FIG. 5. The PCR-amplified DNA fragment was 1,477 bp in size. The amplified DNA fragment was digested with each of *Eco*RI (A) and *Xba*I (B), and the results showed that the band patterns of the susceptible and resistant plants were different from each other. Also, it could be fount that these band patterns were co-segregated with the disease resistance results shown in Example 1.

The results of the PCR amplification performed with the primer combination of SEQ ID NO: 23 and SEQ ID NO: 26 are shown in FIG. 6. The PCR-amplified DNA fragment was 1,846 bp in size. The amplified DNA fragment was digested with *Eco*RI, and the results showed that the band patterns of the susceptible and resistant plants were different from each other. Also, it could be found that these band patterns were co-segregated with the disease resistance results shown in Example 1.

The above results show that, if a primer constructed from a series of any nucleotides selected from the nucleotide sequences set forth in SEQ ID NO: 22 is used to perform PCR and the resulting PCR product is digested with the restriction enzyme present in SEQ ID NO: 22, the presence or absence of a CMV-resistant gene and its genotype (i.e., homo or hetero) can be determined. Namely, since the nucleotide sequences set forth in SEQ ID NO: 22 significantly approach to CMV-resistant genes, the use of any polymorphism shown by the nucleotide sequences allows the presence

or absence of CMV-resistant genes to be determined.

Example 8: Determination of smallest number of consecutive nucleotides which can be converted into CAPS molecular markers

The following test was performed to determine the smallest number of nucleotide sequences convertible into a CAPS molecular marker, in the nucleotide sequences of SEQ ID NO: 22 determined in Example 6. For this purpose, a nucleotide sequence of SEQ ID NO: 23 (forward [SCC07S3]: 5'-GTAGTAGGGTACGGACTCATA-3') was modified with a nucleotide sequence of SEQ ID NO: 27 (forward [SCC07S3-change]: 5'-gGTAGTAGGGTACG-3'), and a nucleotide sequence of SEQ ID NO: 25 (Reverse [CR1541-3]: 5'-GGAGTTTCATCATATGAAGCC-3') was modified with a nucleotide sequence of SEQ ID NO: 28 (Reverse [CR1541-3-change]: 5'-gGGAGTTTCATCAgc-3'). Here, the small letters represent any deletion or substitution. The modified primers of SEQ ID NO: 27 and SEQ ID NO: 28 were used to perform PCR amplification. The PCR amplification consisted of the following: initial denaturation of template DNA for 1 minute at 94 °C; 40 cycles each consisting of 1 minute at 94 °C, 1 minute at 50, 52, 54, 56 or 58 °C, and 2 minutes at 72 °C; and final extension of 10 minutes at 72 °C. The amplified DNA fragment was 1,477 bp in size. Thereafter, the amplified DNA was digested with *Eco*RI and electrophoresed on agarose gel. As shown in FIG. 7, the electrophoresis results showed that the band patterns of the susceptible and resistant plants were different from each other and co-segregated with the disease resistance results shown in Example 1.

The above results suggest that the primer obtained by modifying at least 12 consecutive nucleotides selected from the nucleotide sequences set forth in SEQ ID

NO: 22 is also useful as a CAPS marker for detecting the CMV resistance.

INDUSTRIAL APPLICABILITY

As described above, the present invention provides the molecular marker which comprises the nucleotide sequence highly linkaged to the CMV-resistance, such that CMV-resistant plants can be effectively diagnosed with little or no diagnostic error. Moreover, the inventive molecular marker and the detection method using the same can detect CMV-resistant plants in a rapid and precise manner without inoculating CMV directly into plants, and also can determine the genotype of CMV-resistant plants.